METHOD FOR PRODUCING L-GLUTAMINE AND L-GLUTAMINE PRODUCING BACTERIUM

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to an L-glutamine-producing bacterium belonging to coryneform bacteria, and which is useful for production of L-glutamine. The present inventin also relates to a method for producing L-glutamine. L-glutamine is an industrially useful amino acid as an ingredient in seasonings, liver function promoting agents, amino acid infusions, comprehensive amino acid pharmaceuticals and so forth.

Brief Description of the Related Art

Various techniques for increasing L-amino acid production using recombinant DNA techniques have been disclosed. For example, techniques for enhancing activities of enzymes involved in L-amino acid biosyntheses (WO96/40934), or techniques for reducing activities of enzymes involved in degradation of L-amino acids (WO/96/17930), and so forth, are known. A method for producing L-glutamine using a coryneform bacterium having enhanced glutamine synthetase activity has been disclosed (US Pat App Pub No. 2003/0003550). Furthermore, genes encoding glutamine synthetase (Genbank Accession No. Y13221) and glutamine 2-oxoglutarate aminotransferase (Genbank Accession No. AB024708) have been reported (FEMS Microbiol. Lett. (1997) 154(1) 81-88, Microbiology (2001) 147, 2961-2970), and are known to be involved in glutamine biosynthesis and degradation of coryneform bacteria.

Besides the aforementioned genes, existence of an enzyme involved in degradation of L-

glutamine in coryneform bacteria has been suggested (Amino Acids, 7:73-77 (1963)). However, this enzyme is inhibited by ammonium ions and low pH, and thus barely functions in glutamine fermentation in the presence of the required ammonium ions.

Glutaminase (glutamine amidohydrolase) is known as an enzyme that degrades L-glutamine by hydrolysis. Genes encoding glutaminase have been reported for *Pseudomonas* bacteria (FEMS Microbiol. lett., 178 (2), 327-335 (1999)), *Aspergillus oryzae* (Appl. Microbiol. Biotechnol., 54, 59-68 (2000), EP 1 077 256 A1), *Rhizobium etli* (Biochim. Biophys. Acta, 1444 (3): 451-6, 1999), rat (J. Biol. Chem., 266 (28), 18792-18796 (1991)), among others. Furthermore, existence of glutaminase activity in *Escherichia coli* has been reported (J. Biol. Chem., 243 (5) 853-878 (1968)). However, a glutaminase gene derived from coryneform bacteria has not been identified, and any effect mutation of such a gene will have on glutamine production is not known.

A method for enhancing gene expression by modifying a promoter sequence of the desired gene is known (Japanese Patent Laid-open (Kokai) No. 2000-818935). Furthermore, a gene encoding glutamine synthetase (hereinafter, "*glnA*") from coryneform bacteria is known (FEMS Microbiology Letters, 154, 81-88, 1997). Moreover, the transcription initiation site of the gene including the promoter region has been identified (FEMS Microbiology Letters, 205, 361-367, 2001). However, enhancing the expression of the glutamine synthetase gene by modifying a promoter sequence has not been previously described.

Methods of improving microorganisms by breeding have frequently been used in fermentation production of L-amino acids. That is, since the yield of L-amino acids produced from wild-type microorganisms is often extremely low, methods of imparting auxotrophic or analogue resistance by mutation, or imparting mutations designed to improve metabolic regulation, or a combination of these are known. Although L-glutamine can be obtained by these known methods, there is clearly a need in the art to improve fermentation yields so that production of L-glutamine can

be accomplished efficiently and at a low cost.

SUMMARY OF THE INVENTION

The inventors of the present invention assiduously studied in order to achieve the present invention. The present invention describes a novel gene present in a coryneform bacteria, as well as methods for improved production of L-glutamine using recombinant techniques. Initially, a gene was identified encoding glutaminase, and at the same time, it was discovered that L-glutamine-producing ability was far superior in a strain with reduced glutaminase activity than in a strain where glutaminase activity was similar to that of a wild-type strain. Furthermore, it was discovered that the L-glutamine-producing ability could be further improved by reducing glutaminase activity while simultaneously enhancing glutamine synthetase activity.

An object of the present invention is to improve L-glutamine-producing ability of a coryneform bacterium by reducing L-glutamine degradation ability of the coryneform bacterium, and thereby provide a method for producing L-glutamine by utilizing a bacterial strain having such a characteristic.

It is a further object of the present invention to provide a coryneform bacterium having L-glutamine producing ability and modified so that intracellular glutaminase activity is reduced.

It is a further object of the present invention to provide the bacterium as stated above, wherein the intracellular glutaminase activity is reduced by disrupting a glutaminase gene on a chromosome.

It is even a further object of the present invention to provide the bacterium as stated above, wherein the glutaminase activity is 0.1 U/mg of cellular protein or less.

It is even a further object of the present invention to provide the bacterium as stated above, wherein the glutaminase activity is similar to or less than glutamine synthetase activity when

measured as activity per unit weight of cellular proteins.

It is even a further object of the present invention to provide the bacterium as stated above, which is further modified so that intracellular glutamine synthetase activity is enhanced.

It is even a further object of the present invention to provide the bacterium as stated above, wherein the glutamine synthetase activity is enhanced by increasing the expression of a glutamine synthetase gene.

It is even a further object of the present invention to provide the bacterium as stated above, wherein enhancing the expression of a glutamine synthetase gene is attained by increasing the copy number of the gene encoding glutamine synthetase, or modifying an expression regulatory sequence of the gene encoding glutamine synthetase, so that expression of the gene in the bacterium is enhanced.

It is even a further object of the present invention to provide a method for producing L-glutamine, comprising the steps of culturing a bacterium as stated above in a medium to produce L-glutamine in the medium and collecting the L-glutamine from the medium.

It is even a further object of the present invention to provide a glutamine synthetase gene of a coryneform bacterium, wherein the sequence of -35 region of the gene is replaced with TTGCCA, and the sequence of -10 region of the gene is replaced with TATAAT.

According to the present invention, L-glutamine-producing ability of coryneform bacteria can be improved, resulting in greater yields of L-glutamine produced efficiency and at low cost.

BRIEF DESCRIPTION OF THE DRAWAINGS

Fig. 1 shows scheme for construction of plasmid pHMKGLS5 containing a glutaminase gene.

Fig. 2 shows scheme for construction of plasmid pNEL not containing any region autonomously replicable in coryneform bacteria.

Fig. 3 shows scheme for construction of plasmid pNELΔgls for disruption of *gls*.

Fig. 4 shows scheme for construction of plasmid pNELglnA14 having a GS gene with enhanced expression.

DETTAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Coryneform bacterium of the present invention

In the present invention, coryneform bacteria include, but are not limited to those bacteria classified in the genus *Brevibacterium*, as well as those classified in the genus *Corynebacterium* (Int. J. Syst. Bacteriol., 41, 255 (1981)), which are closely related. Examples of such coryneform bacteria include but are not limited to *Corynebacterium acetoacidophilum, Corynebacterium acetoglutamicum, Corynebacterium alkanolyticum, Corynebacterium callunae, Corynebacterium glutamicum, Corynebacterium lilium, Corynebacterium melassecola, Corynebacterium thermoaminogenes, Corynebacterium herculis, Brevibacterium divaricatum, Brevibacterium flavum, Brevibacterium immariophilum, Brevibacterium lactofermentum, Brevibacterium roseum, Brevibacterium saccharolyticum, Brevibacterium thiogenitalis, Corynebacterium ammoniagenes, Brevibacterium album, Brevibacterium cerium,* and *Microbacterium ammoniaphilum.*

Specifically, the following strains are encompassed: Corynebacterium acetoacidophilum ATCC 13870, Corynebacterium acetoglutamicum ATCC 15806, Corynebacterium alkanolyticum ATCC 21511, *Corynebacterium callunae* ATCC 15991, *Corynebacterium glutamicum* ATCC 13020, 13032, 13060, *Corynebacterium lilium* ATCC 15990, *Corynebacterium melassecola* ATCC 17965, *Corynebacterium thermoaminogenes* AJ12340 (FERM BP-1539), *Corynebacterium herculis* ATCC 13868, *Brevibacterium divaricatum* ATCC 14020, *Brevibacterium flavum* ATCC 13826, ATCC 14067, AJ12418 (FERM BP-2205), *Brevibacterium immariophilum* ATCC 14068, *Brevibacterium lactofermentum* ATCC 13869, *Brevibacterium roseum* ATCC 13825, *Brevibacterium*

saccharolyticum ATCC 14066, *Brevibacterium thiogenitalis* ATCC 19240, *Brevibacterium ammoniagenes* ATCC 6871, ATCC 6872, *Brevibacterium album* ATCC 15111, *Brevibacterium cerium* ATCC 15112, and *Microbacterium ammoniaphilum* ATCC 15354.

These strains can be obtained from, for example, the American Type Culture Collection. Each strain is assigned its accession number, and one can request a desired strain by its accession number. The accession number for each strain is indicated on the catalog of the American Type Culture Collection. The AJ12340 strain was deposited on October 27, 1987 at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (currently, the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, postal code: 305-5466)) as an international deposit under the provisions of the Budapest Treaty, receiving an accession number of FERM BP-1539. The AJ12418 strain was deposited on January 5, 1989 at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry as an international deposit under the provisions of the Budapest Treaty, receiving an accession number of FERM BP-2205.

In the present invention, "L-glutamine-producing ability" means an ability of the coryneform bacterium of the present invention to accumulate L-glutamine in a medium when the bacterium is cultured in the medium. This L-glutamine-producing ability may be a property of a wild-type strain of coryneform bacteria or may be a property imparted or enhanced by breeding.

The method of imparting 6-diazo-5-oxo-norleucine resistance (Japanese Patent Laid-open No. 3-232497), the method of imparting purine analogue resistance and/or methionine sulfoxide resistance (Japanese Patent Laid-open No. 61-202694), the method of imparting α-ketomalonic acid resistance (Japanese Patent Laid-open No. 56-151495), the method of imparting resistance to a

peptide containing glutamic acid (Japanese Patent Laid-open No. 2-186994) and so forth can be used to impart or enhance the L-glutamine-producing ability by breeding. Specific examples of coryneform bacteria having L-glutamine-producing ability include, but are not limited to: *Brevibacterium flavum* AJ11573 (FERM P-5492, refer to Japanese Patent Laid-open No. 56-151495); *Brevibacterium flavum* AJ12210 (FERM P-8123, refer to Japanese Patent Laid-open No. 61-202694); *Brevibacterium flavum* AJ12212 (FERM P-8123, refer to Japanese Patent Laid-open No. 61-202694); *Brevibacterium flavum* AJ12418 (FERM-BP2205, refer to Japanese Patent Laid-open No. 2-186994); *Brevibacterium flavum* DH18 (FERM P-11116, refer to Japanese Patent Laid-open No. 3-232497); *Corynebacterium melassecola* DH344 (FERM P-11117, refer to Japanese Patent Laid-open No. 3-232497); *Corynebacterium glutamicum* AJ11574 (FERM P-5493, refer to Japanese Patent Laid-open No. No. 56-151495).

In addition, the coryneform bacterium of the present invention may be modified so that the intracellular glutaminase activity is reduced. The "glutaminase activity" (hereinafter, "GLS activity") means an enzymatic activity of converting L-glutamine to L-glutamic acid. The GLS activity can be measured, for example, by the following method, among others known in the art.

A crude enzyme solution of coryneform bacterium is added to a solution containing 100 mM Tris-HCl (pH 8.0) and 75 mM L-glutamine, and a reaction is allowed at 30°C for 30 minutes or 60 minutes. Then, SDS is added to the reaction mixture at a final concentration of 0.5% to terminate the reaction, and the resultant L-glutamic acid is quantified. In the present invention, glutaminase activity producing 1 μmol of glutamic acid per minute in the aforementioned reaction system is defined as 1 U. The amount of protein in the crude enzyme solution can be measured by a known method, for example, by using Protein Assay (Bio-Rad) with a bovine serum albumin standard. Hereinafter, the GLS activity per 1 mg of protein is indicated with the unit "U/mg".

The aforementioned crude enzyme solution can be prepared, for example, as follows.

First, to prepare cells, 20 ml of a medium containing 30 g of glucose, 1.5 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 100 μg of $VB_1 \cdot HCl$, 3 μg of biotin, 200 mg of soybean hydrolysates, 1.5 g of urea and 0.02 ml of **anti-foam agent** GD-113 in 1 L of pure water (adjusted to pH 6.8 with NaOH) is introduced into a 500-ml Sakaguchi flask. After sterilization of the medium by autoclave at 115°C for 10 minutes, the cells of the strain are inoculated into the medium and cultured at 31.5°C with shaking at 115 rpm. The culture is finished before the sugar is completely consumed, and the culture broth is instantly cooled. The cells are separated from the culture broth by centrifugation with refrigeration, washed with 100 mM Tris-HCl (pH 8.0) and disrupted by sonication. The undisrupted cells are removed by centrifugation at 15000 g for 15 minute to prepare a crude enzyme solution. The crude enzyme solution is placed on ice until use.

According to the aforementioned method, GLS activities of known L-glutamine-producing bacteria were measured and the results are shown in Table 8.

The expression "modified so that intracellular glutaminase activity is reduced" means that the bacterium has been modified so that the GLS activity per cell becomes lower than that of a wild-type or non-modified strain of coryneform bacterium. Examples include when the number of GLS (glutaminase) molecules per cell decreases, or when GLS activity per GLS molecule decreases and so forth. The "reduction" also includes complete disappearance of activity. A strain or non-modified strain of coryneform bacterium may be compared to, for example, the *Brevibacterium flavum* ATCC 14067. As a result of reduction of GLS activity, the amount of L-glutamine accumulation in a medium increases, and the by-product L-glutamic acid in a medium decreases.

It is sufficient that the coryneform bacterium of the present invention has reduced GLS activity compared with a wild-type strain or non-modified strain. Preferably, the GLS activity as measured in the aforementioned measurement system may be reduced to a level of 0.1 U/mg or less, preferably 0.02 U/mg or less, more preferably 0.01 U/mg or less. However, the present invention is

not limited to the activity of 0.01 U/mg or less.

Although the gene encoding GLS of coryneform bacteria had not been previously identified, the present invention encompasses a gene which was isolated by the inventors from *Brevibacterium flavum*. They searched genes of *Corynebacterium glutamicum*r for a homologous gene to the known GLS gene of *Rhizobium* bacterium (Biochim Biophys Acta, 1444(3): 451-6, Mar. 19, 1999) using the published genome sequence of *Corynebacterium glutamicum* and they found a gene which was estimated to encode GLS. Based on the sequence of the putative GLS gene of *Corynebacterium glutamicum* they isolated GLS gene from *Brevibacterium flavum.*, and which is homologous with the GLS gene of a *Rhizobium* bacterium (Biochim Biophys Acta, 1444(3): 451-6, Mar. 19, 1999). The *gls* gene can be obtained by PCR (see White, T.J. et al., Trends Genet., 5, 185 (1989)) using primers based on that nucleotide sequence, for example, the primers shown in SEQ ID NOS: 5 and 6, and chromosomal DNA of coryneform bacterium as a template. Genes encoding GLS from other microorganisms can also be similarly obtained. The *gls* gene of the *Brevibacterium flavum* ATCC 14067 strain obtained as described above is shown in SEQ ID NO: 1, and the amino acid sequence encoded thereby is shown in SEQ ID NO: 2.

The chromosomal DNA can be prepared from a bacterium serving as a DNA donor by, for example, the method of Saito and Miura (see H. Saito and K. Miura, Biochem. Biophys. Acta, 72, 619 (1963), Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, pp.97-98, Baifukan, 1992) and the like.

Methods for reducing the GLS activity of coryneform bacteria include, but are not limted to, for example, treating the bacteria by ultraviolet irradiation, or with a mutagenizing agent, followed by selection of the resultant mutant strain. Mutagenizing agents which are useful in the present invention include N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or nitrous acid. Methods other than mutagenesis treatments for obtaining a coryneform bacteria having reduced GLS activity include gene

disruption. That is, a coryneform bacterium is transformed with a DNA containing a *gls* gene whereby a partial sequence of the *gls* gene is deleted so as to disrupt GLS function (deletion- type *gls* gene), and the subsequent recombination between the deletion-type *gls* gene and the *gls* gene on the chromosome disrupts the *gls* gene on the chromosome. Such gene disruption by gene substitution via homologous recombination is known, as well as methods of utilizing linear DNA, methods of utilizing a plasmid containing a temperature sensitive replication origin, and so forth.

Attenuation of the GLS activity can also be accomplished by replacing an expression regulatory sequence such as *gls* gene promoter with a weaker one (Japanese Patent Laid-open No. 2000-818935). The mutatagenesis methods and the gene disruption methods may be used in combination.

A *gls* gene on a host chromosome can be replaced with the deletion-type *gls* gene, for example, in the following manner. A recombinant DNA is prepared by inserting a temperature-sensitive replication origin, the deletion-type *gls* gene and a marker gene for resistance to a drug such as chloramphenicol, and used to transform a coryneform bacterium. Then, the resultant transformant is cultured in the medium containing the drug so that the temperature-sensitive replication origin does not function, and, as a result, a transformant is obtained in which the recombinant DNA has been incorporated into the chromosomal DNA.

To introduce the recombinant DNA prepared as described above into a coryneform bacterium, any known transformation method can be employed. For instance, such methods include treating recipient cells with calcium chloride so as to increase the permeability of the cells for DNA, which has been reported for *Escherichia coli* K-12 (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)), and preparing competent cells from cells which are at the growth phase followed by introducing the DNA thereinto, which has been reported for *Bacillus subtilis* (Duncan, C.H., Wilson, G.A. and Young, F.E., Gene, 1, 153 (1977)). Furthermore, a method of making DNA-recipient cells

into protoplasts or spheroplasts, which can easily take up recombinant DNA, followed by introducing the recombinant DNA into the DNA-acceptor cells, which is known to be applicable to *Bacillus subtilis*, actinomycetes and yeasts (Chang, S. and Choen, S.N., Molec. Gen. Genet., 168, 111 (1979); Bibb, M.J., Ward, J.M. and Hopwood, O.A., Nature, 274, 398 (1978); Hinnen, A., Hicks, J.B. and Fink, G.R., Proc. Natl. Sci., USA, 75, 1929 (1978)) can be also employed. The transformation of coryneform bacteria can also be performed by the electric pulse method (Sugimoto et al., Japanese Patent Laid-open No. 2-207791).

Examples of the temperature-sensitive plasmid for coryneform bacteria include, but are not limited to p48K and pSFKT2 (see Japanese Patent Laid-open No. 2000-262288 for these), pHSC4 (see France Patent Laid-open No. 2667875, 1992 and Japanese Patent Laid-open No. 5-7491) and so forth. These plasmids can autonomously replicate at 25°C at least, but cannot autonomously replicate at 37°C. The *Escherichia coli* AJ12571 harboring pHSC4 was deposited at the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology, the Ministry of International Trade and Industry (currently, the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, postal code: 305-5466)) on October 11, 1990, receiving an accession number of FERM P-11763. Then, the deposit was converted into an international deposit under the provisions of the Budapest Treaty on August 26, 1991, receiving an accession number of FERM BP-3524. Furthermore, it is also possible to transform a coryneform bacterium with a plasmid that cannot autonomously replicate in coryneform bacteria and incorporate the plasmid into the chromosome of the coryneform bacterium by homologous recombination, as described in the examples section.

In a strain incorporating recombinant DNA into chromosomal DNA as described above, the deletion-type *gls* is recombined with the native *gls* sequence present on the chromosome, and the

chromosomal *gls* and the deletion-type *gls* are inserted into the chromosome so that the other portions of the recombinant DNA (vector segment, temperature sensitive replication origin and drug resistance marker) are present between the two genes. Therefore, the transformant strain expresses native *gls*, because the native *gls* is dominant in this state.

Then, so that only the deletion-type *gls* remains on the chromosomal DNA, one copy of *gls* is eliminated from the chromosomal DNA with the vector segment (including the temperature sensitive replication origin and the drug resistance marker) by recombination of two of *gls* genes. In this case, the normal *gls* is left on the chromosomal DNA, and the deletion-type *gls* is excised from the chromosomal DNA, or vice versa. In the both cases, the excised DNA may be retained in the cell as a plasmid when the cell is cultured at a temperature at which the temperature sensitive replication origin can function. Subsequently, if the cell is cultured at a temperature at which the temperature sensitive replication origin cannot function, *gls* on the plasmid is eliminated along with the plasmid from the cell. Then, a strain in which *gls* is disrupted can be obtained by selecting a strain in which the deletion-type *gls* is left on the chromosome by using PCR, Southern hybridization or the like.

It is sufficient that the deletion-type *gls* gene used for the gene disruption should have a homology to the target *gls* gene on the chromosomal DNA of the coryneform bacterium to such a degree that homologous recombination results. The homology is preferably 70% or more, more preferably 80% or more, still more preferably 90% or more, and most preferably 95% or more. Furthermore, DNAs that can hybridize with each other under stringent conditions may cause homologous recombination. The "stringent conditions" include conditions under which a so-called specific hybrid is formed, and a non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include conditions whereby DNAs hybridize with each other at a salt concentration typically used for washing

in Southern hybridization, i.e., 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C.

It is assumed that in the cells of coryneform bacteria, L-glutamine is synthesized by glutamine synthetase ("GS"), and degraded by glutaminase ("GLS"). That is, the inventors of the present invention deduced that to efficiently produce L-glutamine in high yields, it was important to maintain the GS activity at a high level, while keeping the GLS activity at a low level. However, in strains of coryneform bacteria, the GS activity is remarkably lower than the GLS activity. It is needless to say that, although the equilibrium of intracellular L-glutamine formation and degradation in cells during L-glutamine production is changed with not only specific activities of these enzymes, but also with Km values of the enzymes and intracellular concentrations of substrates, the specific activities represent an important factor.

For example, as described in Example 4, in a mutant strain having reduced GLS activity, the L-glutamine yield is increased when the residual GLS activity is suppressed to a level of about 60 percent of the GS activity.

The GS activity can be measured, for example, as follows. The reaction by GS can be quantified by adding a crude enzyme solution of a coryneform bacterium to a solution containing 100 mM imidazole-HCl (pH 7.0), 90 mM KCl, 0.1 mM NH_4Cl, 1 mM $MnCl_2$, 1 mM phosphoenolpyruvic acid, 0.3 mM NADH, 10 U of lactate dehydrogenase, 25 U of pyruvate kinase, 1 mM ATP and 10 mM MSG (sodium glutamate), and measuring variation of absorbance at 340 nm at 30°C. For the measurement of blank, the aforementioned reaction solution without the MSG is used. The protein concentration of the crude enzyme solution is quantified by using Protein Assay (Bio-Rad) with bovine serum albumin as a standard sample. In the present invention, the amount of enzyme necessary to produce 1 μmol of NAD per minute in the aforementioned reaction system is defined as 1 U. Hereafter, the GS activity per 1 mg of protein is indicated with a unit of "U/mg".

The aforementioned crude enzyme solution is prepared, for example, as follows. That is,

the crude enzyme solution is prepared by separating cells from the culture broth by centrifugation, washing the cells with 100 mM imidazole-HCl (pH 7.0, a solution containing 90 mM KCl), sonicating the cells and removing insoluble fraction by ultracentrifugation.

In order to efficiently produce L-glutamine by using the coryneform bacterium of the present invention, a strain in which the GLS activity is reduced and the glutamine synthetase activity is enhanced simultaneously is preferably used.

The expression "glutamine synthetase activity is enhanced" means that the GS activity per cell is higher than that of a coryneform bacterium. For example, it can be exemplified by a case where the number of GS molecules per cell increases, and a case where the GS activity per GS molecule increases and so forth. Furthermore, a coryneform bacterium that serves as an object for comparison may be, for example, the *Brevibacterium flavum* ATCC 14067. As a result of enhancement of the GS activity, the amount of L-glutamine accumulation in a medium increases, and the L-glutamic acid by-product decreases, and so forth, can be obtained.

It is sufficient that in the coryneform bacterium of the present invention, the GLS activity is reduced, and the GS activity is enhanced as compared with a strain or unmodified strain. Preferably, the coryneform bacterium of the present invention is a bacterium in which the GLS activity should be similar to or less than the GS activity, more preferably the GLS activity should be 1/2 or less of the GS activity, when measured as activity per unit weight of cellular proteins. In the present invention, the GLS activity and GS activity per unit weight of cellular protein mean the activities measured by the aforementioned measurement methods and defined according to the aforementioned definitions.

Enhancing the GS activity in a coryneform bacterium can be attained by increasing the copy number of the gene encoding GS. For example, a recombinant DNA can be prepared by ligating a gene fragment encoding GS with a vector known to function in the bacterium, preferably a multi-copy type vector, and transform the vector into a host having L-glutamine-producing ability. Alternatively,

the aforementioned recombinant DNA can be introduced into a coryneform bacterium to obtain a transformant, and the L-glutamine-producing ability can be subsequently imparted to the transformant.

Any of genes derived from coryneform bacteria, or genes derived from other organisms such as bacteria belonging to the genus *Escherichia* can be used as the GS gene. Among these, genes derived from coryneform bacteria are preferred for their ease of expression.

The *glnA* gene is known to encode GS of coryneform bacterium (FEMS Microbiology Letters, 154, 81-88, 1997). The GS gene can be obtained by PCR using primers based on the known nucleotide sequence, for example, the primers shown in SEQ ID NOS: 19 and 20, and using chromosomal DNA of a coryneform bacterium as a template. Genes encoding GS from other microorganism can also be similarly obtained. The nucleotide sequence of the *glnA* gene of the *Brevibacterium flavum* ATCC 14067 strain and the amino acid sequence encoded thereby are shown in SEQ ID NOS: 3 and 4.

The gene encoding GS may be, besides the *glnA* gene, one encoding an amino acid sequence which includes substitution, deletion, insertion, addition or inversion of one or several amino acids, so long as the encoded GS has the activity of catalyzing the reaction of L-glutamic acid and ammonium ion to produce L-glutamine. The number of "several" amino acids as used herein varies depending on the positions of amino acid residues in the three-dimensional structure of the protein and the types of the amino acids. However, it preferably means between 2 to 30, more preferably between 2 to 20, and most preferably between 2 to 10.

Examples of DNA encoding substantially the same protein as GS described above include DNA which is hybridizable with a probe having the sequence of nucleotide numbers of 874 to 2307 of the nucleotide sequence shown in SEQ ID NO: 3 or a probe prepared from this nucleotide sequence under stringent conditions, and encodes a protein having an activity similar to GS. The "stringent conditions" include conditions in which a so-called specific hybrid is formed, and a non-

specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include a condition whereby DNAs having high homology, for example, DNAs having homology of 70% or more, preferably 80% or more, more preferably 90% or more, still more preferably 95% or more , hybridize with each other, whereas DNAs having homology lower than the above do not hybridize with each other. Alternatively, the stringent conditions are exemplified by conditions whereby DNAs hybridize with each other at a salt concentration typical for washing in Southern hybridization, i.e., 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C.

A vector that functions in coryneform bacteria means, for example, a plasmid that can autonomously replicate in coryneform bacteria. Specific examples thereof include, but are not limited to the pAM330 (see Japanese Patent Laid-open No. 58-67699), pHM1519 (see Japanese Patent Laid-open No. 58-77895), pSFK6 (see Japanese Patent Laid-open No. 2000-262288)

If a DNA fragment having an ability to make a plasmid autonomously replicable in coryneform bacteria is removed from these vectors and inserted into the aforementioned vectors for *Escherichia coli*, they can be used as a so-called shuttle vector autonomously replicable in both of *Escherichia coli* and coryneform bacteria.

Examples of such a shuttle vector include, but are not limited to those described herein. Bacterial strains that harbor each of the vectors and accession numbers thereof at the international depositories in the parentheses are as follows:

pAJ655 *Escherichia coli* AJ11882 (FERM BP-136)

Corynebacterium glutamicum SR8201 (ATCC 39135)

pAJ1844 *Escherichia coli* AJ11883 (FERM BP-137)

Corynebacterium glutamicum SR8202 (ATCC 39136)

pAJ611 *Escherichia coli* AJ11884 (FERM BP-138)

pAJ3148 *Corynebacterium glutamicum* SR8203 (ATCC 39137)

pAJ440 *Bacillus subtilis* AJ11901 (FERM BP-140)

pHC4 *Escherichia coli* AJ12617 (FERM BP-3532)

These vectors can be obtained from the deposited bacteria as follows. That is, cells collected in their exponential growth phase are lysed using lysozyme and SDS, and centrifuged at 30000 x g. The supernatant obtained from the lysate is added with polyethylene glycol, fractionated and purified by cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

The copy number of the GS gene can also be increased by allowing multiple copies of the gene to exist on chromosomal DNA of a coryneform bacterium. This can be performed by targeting a sequence present on chromosomal DNA in multiple copy number. A repetitive DNA or an inverted repeat present at the end of a transposable element can be used as the sequence present on chromosomal DNA in multiple copy number. Alternatively, as disclosed in Japanese Patent Laid-open No. 2-109985, multiple copies of the GS gene can be introduced into chromosomal DNA by incorporating them into a transposon and transferring it.

Besides the above gene amplification methods, expression of the GS gene can be enhanced by replacing an expression control sequence, such as promoters of GS gene, with a stronger one. Examples of strong promoters include *lac* promoter, *trp* promoter, *trc* promoter and so forth. Moreover, it is also possible to introduce nucleotide substitutions for several nucleotides into a promoter region of the GS gene so that it is modified into a stronger one, as disclosed in International Patent Publication WO00/18935. By such substitution or modification of the promoter region, expression of the GS gene is enhanced, and thus the GS activity is enhanced. Such modification of expression regulatory sequence may be combined with the increase of copy number of the GS gene.

For example, the transcription initiation site of the *glnA* gene of coryneform bacteria as well as its promoter region have been reported by Anton et al. (FEMS Microbiogy Letters, 205, 361-367,

2001). For example, the GS activity is enhanced by replacing the sequence of -10 region with TATAAT, and -35 region of the GS gene with TTGCCA, which regions are disclosed in Fig. 3C of the aforementioned reference. However, in the present invention, the -35 to -30 region of the GS gene means a site which is located 3 bp downstream, i.e., toward transcription initiation site, from -35 to -30 region described in the aforementioned reference (nucleotide numbers 727 to 732 in SEQ ID NO: 3). On the other hand, the -10 region of the GS gene means a site which is the same as -10 region described in the aforementioned reference (nucleotide numbers 751 to 756 in SEQ ID NO: 3). Such modifications of these regions of the GS gene can be performed by, for example, site-specific mutagenesis.

Examples of the GS gene where the -35 region and -10 region are modified include, but are not limited to the *glnA* gene of coryneform bacteria, for example, a gene having the sequence of SEQ ID NO: 3. The encoded protein may have an amino acid sequence including substitution, deletion, insertion, addition, or inversion of one or several amino acid residues so long as the encoded protein has an activity of catalyzing the reaction generating L-glutamine from an ammonium ion and L-glutamic acid.

Besides increase of expression of the GS gene described above, the GS activity can also be enhanced by eliminating the activity control via the adenylylation of intracellular GS (US Pat App Pub No. 2003/0003550A1)

In the coryneform bacterium of the present invention, besides reduction of the GLS activity and enhancement of the GS activity, an activity of an enzyme catalyzing a reaction involved in the L-glutamine biosynthesis pathway may be enhanced. Examples of the such an enzyme include, but are not limited to isocitrate dehydrogenase, aconitate hydratase, citrate synthase, pyruvate dehydrogenase, phosphoenolpyruvate carboxylase, pyruvate carboxylase, pyruvate kinase, phosphofructokinase and so forth.

Furthermore, activity of an enzyme that catalyzes a reaction branching off from the L-glutamine biosynthesis pathway and producing a compound other than L-glutamine may be reduced or eliminated. Examples of such an enzyme include, but are not limited to isocitrate lyase, α-ketoglutarate dehydrogenase, glutamate synthase and so forth.

Production of L-glutamine using microorganism of the present invention

L-glutamine can be efficiently produced and the L-glutamic acid by-product can be suppressed by culturing a coryneform bacterium obtained as described above in a medium. L-glutamate is produced, and accumulates in the medium and may be collected from the medium.

In order to produce L-glutamine by using the coryneform bacterium of the present invention, culture can be performed in a conventional manner using a typical medium containing a carbon source, nitrogen source and mineral salts as well as organic trace nutrients such as amino acids and vitamins, as required. Either a synthetic medium or a natural medium may be used. Any kind of carbon source and nitrogen source may be used so long as they can be utilized by the strain to be cultured.

Sugars such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysates and molasses, and organic acids such as acetic acid and citric acid, and alcohols such as ethanol can also be used each alone or in a combination with other carbon sources as the carbon source.

Ammonia, ammonium salts such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate and ammonium acetate, nitric acid salts and so forth can be used as the carbon source.

Amino acids, vitamins, fatty acids, nucleic acids, those containing those substances such as peptone, casamino acid, yeast extract and soybean protein decomposition product and so forth can be

used as the carbon source. It is preferable to supplement the required nutrient when an auxotrophic mutant strain that requires an amino acid or the like for its growth is used.

Phosphoric acid salts, magnesium salts, calcium salts, iron salts, manganese salts and so forth can be used as the mineral salts.

The culture may be performed with aeration. The culture temperature is controlled to be 20 to 45°C, and pH is controlled to be 5 to 9. When pH falls during the culture, the medium can be neutralized by addition of calcium carbonate or an alkali such as ammonia gas or the like. A substantial amount of L-glutamine is accumulated in the culture broth after 10 to 120 hours of culture in such a manner as described above.

L-glutamine can usually be collected from the culture broth after the culture in a conventional manner. For example, after the cells were removed from the culture broth, L-glutamine can be collected by concentrating the broth to crystallize L-glutamine.

Examples

Hereinafter, the present invention will be explained more specifically with reference to the preferred embodiments, given only by way of example.

Example 1: Constructions of *gls*-amplified strain

(1) Measurement of glutaminase activity of coryneform bacterium

Existence of glutaminase, an enzyme that degrades L-glutamine to generate L-glutamic acid, had not been definitely reported in coryneform bacteria. Therefore, the inventors of the present invention verified whether the glutaminase activity existed in coryneform bacteria.

The *Brevibacterium flavum* ATCC 14067 strain was inoculated into a medium containing 30 g of glucose, 1.5 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 100 μg of

VB_1•HCl, 3 μg of biotin, 200 mg of soybean hydrolysates, 1.5 g of urea and 0.02 ml of anti-foam agent GD-113 in 1 L of pure water (adjusted to pH 7.0 with KOH) and cultured at 31.5°C with shaking. The cells were separated from the culture broth by centrifugation, washed with 100 mM Tris-HCl (pH 8.0) and disrupted by sonication. The undisrupted cells were removed by centrifugation to obtain a crude enzyme solution. The protein concentration in the crude enzyme was quantified by using Protein Assay (Bio-Rad) with bovine serum albumin as a standard sample.

The glutaminase activity was measured by adding the crude enzyme solution to a solution containing 100 mM Tris-HCl (pH 8.0) and 75 mM L-glutamine, allowing a reaction at 30°C for 30 minutes or 60 minutes, then adding SDS at a final concentration of 0.5% to terminate the reaction, and quantifying the produced L-glutamic acid. As a result, it was demonstrated that an enzyme exhibiting the glutaminase activity was present in *Brevibacterium flavum* as shown in Table 1.

Table 1: GLS activity of coryneform bacterium

Strain	GLS (U/mg)
ATCC 14067	0.25

(2) Cloning of glutaminase gene

It is known that L-glutamine serves as a donor of NH_3 in the biosyntheses of nucleic acids, amino acids etc., and that the small subunit of carbamoylphosphate synthetase exhibits the glutaminase activity. Furthermore, a gene encoding glutaminase, *glsA*, was recently cloned for *Rhizobium etli* and characterized as providing the glutaminase activity independently from the nucleic acid and amino acid biosyntheses (Biochim. Biophys. Acta., 1444 (3): 451-6, 1999). Therefore, the inventors of the present invention searched genes of *Corynebacterium glutamicum* for a homologous gene to *glsA*, and as a result, they found a gene which was estimated to encode GLS . Then, based on the nucleotide sequence of the putative GLS gene, the homologue of the gene was cloned and

amplified in *Brevibacterium flavum*, and improved glutaminase activity was verified. The nucleotide sequence of the cloned gene is shown in SEQ ID NO: 1 (hereafter, a gene having the nucleotide sequence of SEQ ID NO: 1 is referred to as "*gls*").

The target sequence was amplified by PCR using the primers shown in SEQ ID NOS: 5 and 6, and using the chromosomal DNA of *Brevibacterium flavum* ATCC 14067 strain as a template. The sequences of SEQ ID NOS: 5 and 6 correspond to the nucleotide numbers 1 to 20 and 2100 to 2081 of SEQ ID NO: 1, respectively. The chromosomal DNA of *Brevibacterium flavum* ATCC 14067 strain was prepared using Bacterial Genome DNA Purification Kit (Advanced Genetic Technologies Corp.). PCR was performed for 30 cycles each consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 3 minutes by using Pyrobest DNA Polymerase (Takara Shuzo).

The resulting PCR product was purified in a conventional manner and blunt-ended using Blunting Kit (Takara Shuzo). The blunt-ended PCR product was ligated by using Ligation Kit (Takara Shuzo) with a shuttle vector for coryneform bacteria and *Escherichia coli*, pHMK2, which had been digested with *Sma*I. The ligation mixture was used to transform competent cells of *Escherichia coli* JM109 (Takara Shuzo). The cells were plated on L medium containing 10 μg/ml of IPTG, 40 μg/ml of X-Gal and 25 μg/ml of kanamycin and cultured overnight. Then, the emerged white colonies were selected and separated into single colonies to obtain transformants. Plasmids are prepared from the transformants by the alkali method, and a plasmid in which the objective PCR fragment was inserted into the vector was isolated. The obtained plasmid was designated as pHMKGLS5.

The aforementioned pHMK2 was obtained as follows. The plasmid pHK4 (see Japanese Patent Laid-open No. 5-7491) having a replication origin derived from the plasmid pHM1519 autonomously replicable in coryneform bacteria (Agric. Biol. Chem., 48, 2901-2903 (1984)) was

digested with the restriction enzymes *Bam*HI and *Sma*I to obtain a fragment containing the replication origin. The obtained fragment was blunt-ended by using DNA Blunting Kit (Takara Shuzo), and inserted into the *Bsa*AI site of the cloning vector pK1 for *E. coli* (Japanese Patent Laid-open No. 2000-262288) to obtain pHMK2. The construction process of pHMKGLS5 is shown in Fig. 1.

(3) Construction of *gls*-overexpressing strain

pHMGLS5 obtained in (2) described above was introduced into a coryneform bacterium to obtain a *gls*-amplified strain. Specifically, the *Brevibacterium flavum* ATCC 14067 strain was transformed with pHMGLS5 by the electric pulse method (see Japanese Patent Laid-open No. 2-207791), plated on CM2G medium (10 g/L of polypeptone, 10 g/L of yeast extract, 5 g/L of NaCl, 1 g/L of glucose, pH 7.0 (KOH)) containing 25 μg/ml of kanamycin, and cultured at 31.5°C for two days. The emerged colony was isolated as a transformant and designated as 2247/pHMKGLS5. A pHMK2-introduced strain was also constructed, and the obtained transformant was designated as 2247/pHMK2. These transformants were each cultured by the method described in (1), and the GLS activity was measured. As a result, it was confirmed that the glutaminase activity increased in the pHMGLS5-introduced strain (Table 2). The plasmid-harboring ratio of the transformants was 100%.

Table 2: GLS activity of GLS-amplified strain

Strain	GLS (U/mg)
ATCC14067/pHMK2	0.19
ATCC14067/pHMKGLS5	0.33

Example 2: Construction of *gls*-deficient strain

(1) Construction of plasmid for disruption of *gls*

In order to confirm whether any gene encoding glutaminase of coryneform bacteria other

than the *gls* gene might exist, a *gls*-deficient strain was constructed. The method is specifically described herein.

First, PCR was performed by using the chromosomal DNA of *Brevibacterium flavum* ATCC 14067 strain as a template and the synthetic DNAs of SEQ ID NOS: 5 and 7 as primers to obtain an amplification product of the N-terminus side of the *gls* gene. Then, in order to obtain an amplification product of a sequence of the C-terminus side of the *gls* gene, PCR was performed using the chromosomal DNA of *Brevibacterium flavum* ATCC 14067 strain as a template and the synthetic DNAs of SEQ ID NOS: 6 and 8 as primers. The sequences of SEQ ID NOS: 7 and 8 are partially complementary to each other. The sequences of SEQ ID NOS: 7, 8, 9, and 10 correspond to the nucleotide numbers 1245 to 985, 983 to 1245, 414 to 438, and 1869 to 1845 of SEQ ID NO: 1, respectively, and the sequences of SEQ ID NOS: 7 and 8 are deficient in the nucleotides of the nucleotide numbers 1003 to 1230 of SEQ ID NO: 1. PCR was performed by using Z-Taq (Takara Shuzo) for 30 cycles each consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 30 seconds.

Then, in order to obtain a *gls* gene fragment having an internal sequence deleted, the aforementioned gene fragments of the N- and C-terminus sides were mixed in substantially equimolar amounts, and PCR was performed using this mixture as a template and the synthetic DNAs of SEQ ID NOS: 9 and 10 as primers to obtain a *gls* gene amplification product having a mutation. PCR was performed using Z-Taq (Takara Shuzo) for 30 cycles each consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 30 seconds. This *gls* gene product has the amino acid sequence of SEQ ID NO: 2 whereby the 110th to 185th amino acids are deleted.

The resultant PCR product was purified in a conventional manner, then digested with *Sma*I and inserted into the *Sma*I site of pNEL. By using this DNA, competent cells of *Escherichia coli*

DH5α (Takara Shuzo) were transformed, and the cells were applied to L medium containing 40 μg/ml of X-Gal and 25 μg/ml of kanamycin and cultured overnight. Then, the emerged blue colonies were picked up, and separated into single colonies to obtain transformant strains. Plasmids were extracted from the obtained transformants, and the plasmid inserted with the target PCR product was designated as pNELΔgls.

pNEL is a plasmid obtained by performing PCR using pNEOL (see WO00/18935) as a template and the synthetic DNAs shown in SEQ ID NOS: 11 and 12 as primers, digesting the amplification product with *Sma*I and self-ligating the digestion product. The plasmid pNEL does not contain any region autonomously replicable in the cells of coryneform bacteria. PCR was performed by using Pyrobest DNA Polymerase (Takara Shuzo) for 30 cycles each consisting of denaturation at 98°C for 20 seconds, annealing and extension at 68°C for 6 minutes. The construction scheme of pNEL is shown in Fig. 2, and the construction scheme of pNELΔgls is shown in Fig. 3.

(2) Construction of *gls*-deficient strain

pNELΔgls obtained in (1) does not contain a region autonomously replicable in the cells of coryneform bacteria. Therefore, if a coryneform bacterium is transformed with this plasmid, a strain having this plasmid introduced into the chromosome by homologous recombination would appear as a transformant although it occurs at an extremely low frequency. The *Brevibacterium flavum* ATCC 14067 strain was transformed with a high concentration of pNELΔgls by the electric pulse method, plated on CM2G medium (10 g/L of polypeptone, 10 g/L of yeast extract, 5 g/L of NaCl, 1 g/L of glucose, pH 7.0 (KOH)) containing 25 μg/ml of kanamycin, and cultured at 31.5°C for two days, and the emerged colony was isolated as a transformant. This transformant forms a blue colony on the CM2G plate containing 40 μg/ml of X-Gal. Then, this transformant was subcultured in CM2G

medium not containing kanamycin, appropriately diluted and then applied to a CM2G plate containing 40 μg/ml of X-Gal. From a large number of emerged colonies, the strains that formed a white colony and showed kanamycin (km) sensitivity were chosen.

PCR was performed using the chromosomal DNA of these Km sensitive strains as a template and synthetic DNAs of SEQ ID NOS: 5 and 6 as primers, and a strain that provided a PCR product having a size smaller than that obtained using the chromosomal DNA of ATCC 14067 as a template was used for the following experiments as a *gls*-deficient strain (hereinafter, 2247Δgls).

(3) Introduction of *gls* plasmid into 2247Δgls

The plasmid pGLS5 described in Example 1, section (2) was introduced into the 2247Δgls strain obtained in (2) described above by the electric pulse method, and a transformant was obtained by using the kanamycin resistance as a marker. The obtained transformant was designated as 2247Δgls/pGLS5. Separately, pHMK2 was also introduced, and the obtained transformant was designated as 2247Δgls/pHMK2.

(4) Measurement of GLS activity of *gls*-deficient strain

The result of GLS activity measurement performed by the method described in Example 1, (1) for *Brevibacterium flavum* ATCC 14067, 2247Δgls and pHMK2 and pHMKGLS-introduced strains thereof are shown in Table 3. It was confirmed that the activity to degrade L-glutamine had almost disappeared in 2247Δgls. Furthermore, the disappearance of the activity was complemented by the introduction of pHMKGLS5. Thus, it was deduced that the gene mainly responsible for the glutaminase activity of coryneform bacteria is *gls*.

Table 3: GLS activity of GLS-deficient strain
and strain complemented with plasmid

Strain	GLS activity(U/mg)
2247Δgls	0.003
2247Δgls/pHMK2	0.000
2247Δgls/pHMKGLS5	0.19

Example 3: Production of L-glutamine by *gls*-deficient strain

(1) Evaluation of culture of *gls*-deficient strain

L-glutamine was produced by *Brevibacterium flavum* ATCC 14067 strain and 2247Δgls strain as follows. Cells of the *Brevibacterium flavum* ATCC 14067 strain and the 2247Δgls strain obtained by culture on a CM2B plate medium were each inoculated into a medium containing 100 g of glucose, 60 g or 40 g of $(NH_4)_2SO_4$, 2.5 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 350 μg of $VB_1 \cdot HCl$, 4 μg of biotin, 200 mg of soybean hydrolysates and 50 g of $CaCO_3$ in 1 L of pure water (adjusted to pH 6.8 with NaOH) and cultured at 31.5°C with shaking until the sugar in the medium was consumed.

After the completion of the culture, the amount of accumulated L-glutamine in the culture broth was analyzed for appropriately diluted culture broth by liquid chromatography. CAPCELL PAK C18 (Shiseido) was used as a column, and the sample was eluted with an eluent containing 0.095% phosphoric acid, 3.3 mM heptanesulfonic acid and 5% acetonitrile in 1 L of distilled water. The accumulated L-glutamine (Gln) amount was analyzed based on variation of absorbance at 210 nm. Furthermore, the accumulated L-glutamic acid (Glu) amount was analyzed for appropriately diluted culture broth by using Biotech Analyzer AS210 (Asahi Chemical Industry). The results of the above analysis are shown in Table 4 (60 g/L of ammonium sulfate) and Table 5 (40 g/L of ammonium sulfate).

As for the 2247Δgls strain, about 3% of improvement in the yield was recognized for all of the conditions compared with the parent strain, the ATCC 14067 strain. These results demonstrated that disappearance or reduction of the GLS activity was effective for production of L-glutamine.

Table 4: L-Glutamine production by GLS activity-reduced strain (1)

Strain	OD_{620} (X101)	Gln (g/L)	Glu (g/L)	Gln yield (%)
ATCC 14067	0.398	9.9	33.4	9.7
2247Δgls	0.427	13.2	30.0	12.8

Table 5: L-Glutamine production by GLS activity-reduced strain (2)

Strain	OD_{620} (X101)	Gln (g/L)	Glu (g/L)	Gln yield (%)
ATCC 14067	0.489	6.8	42.6	6.4
2247Δgls	0.492	10.1	37.6	9.5

Example 4: Construction of *gls*-deficient and GS activity-enhanced strain

(1) Construction of plasmid having GS gene with enhanced expression

The nucleotide sequence of the GS gene of coryneform bacteria has been already elucidated (Genbank Accession No. Y13221). By referring to this sequence, a GS gene with enhanced expression (enhanced-type GS gene) was constructed. The method will be specifically described herein. First, primary PCR was performed for the N-terminus side using the chromosomal DNA of *Brevibacterium flavum* ATCC 14067 strain as a template and DNAs of SEQ ID NOS: 13 and 18 as primers, and primary PCR for the C-terminus side was performed using DNAs of SEQ ID NOS: 15 and 17 as primers. The sequences of SEQ ID NOS: 13, 14, 15, 16, 17, and 18 correspond to the nucleotide numbers 487 to 507, 523 to 549, 1798 to 1775, 1770 to 1745, 1118 to 1169, and 1169 to 1118 of Genbank Accession No. Y13221, respectively. The sequences of SEQ ID NOS: 17 and 18 are complementary to each other. PCR was performed by using Pyrobest DNA Polymerase (Takara Shuzo) for 30 cycles each consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 1 minute.

Then, in order to obtain an enhanced type GS gene fragment, the aforementioned amplification products of the upstream side and downstream side of the GS gene were mixed in

substantially equimolar amounts, and PCR was performed using this mixture as a template and the synthetic DNAs of SEQ ID NOS: 14 and 16 as primers to obtain a GS gene amplification product having a mutation. PCR was performed by using Pyrobest DNA Polymerase (Takara Shuzo) for 30 cycles each consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 2 minutes.

Then, the PCR product was purified in a conventional manner, then digested with *Sma*I and inserted into the *Sma*I site of pNEL. Competent cells of *Escherichia coli* DH5α (Takara Shuzo) were transformed with the obtained DNA, and the cells were applied to L medium containing 40 μg/ml of X-Gal and 25 μg/ml of kanamycin and cultured overnight. Then, the emerged blue colonies were picked up, and separated into single colonies to obtain transformants. Plasmids were extracted from the obtained transformants, and the *glnA* expression regulatory region was sequenced. The plasmid introduced with the objective mutation was designated as pNELglnA14. The construction scheme of pNELglnA14 is shown in Fig. 4. In the *glnA* gene fragment cloned in pNELglnA14 obtained as described above, the sequence (ATTATA) of the region downstream by 3 bp from the -35 site of the GS gene described in FEMS Microbiogy Letters, 205 (2001) 361-367 is replaced with TATAAT, and the sequence (TTTTGA) of the –10 site is replaced with TTGCCA.

(2) Construction of GS activity-enhanced strain

pNELglnA14 obtained (1) described above does not contain any region autonomously replicable in the cells of coryneform bacteria. Therefore, if a coryneform bacterium is transformed with this plasmid, a strain in which this plasmid is introduced into the chromosome by homologous recombination would appear as a transformant although it occurs at an extremely low frequency. The *Brevibacterium flavum* 2247Δgls strain was transformed with a high concentration of pNELglnA14 by the electric pulse method, plated on CM2G medium (10 g/L of polypeptone, 10 g/L

of yeast extract, 5 g/L of NaCl, 1 g/L of glucose, pH 7.0 (KOH)) containing 25 μg/ml of kanamycin and cultured at 31.5°C for two days, and the emerged colonies were isolated as transformants. These transformants form a blue colony on the CM2G plate containing 40 μg/ml of X-Gal. Then, these transformants were subcultured in CM2G medium not containing kanamycin (Km), appropriately diluted and then applied to a CM2G plate containing 40 μg/ml of X-Gal. From a large number of emerged colonies, the strains that formed a white colony and showed kanamycin susceptibility were chosen.

PCR was performed using the chromosomal DNA of these Km sensitive strains as a template and the synthetic DNAs of SEQ ID NOS: 14 and 16 as primers, and the expression regulatory region was sequenced. Among the Km sensitive strains, a strain in which the objective mutation was inserted into the expression regulatory region was isolated. The obtained strain was designated as 2247ΔglsglnA14 and used for the following experiments.

(3) Measurement of GS activity

The *Brevibacterium flavum* ATCC 14067 strain was inoculated into a medium containing 30 g of glucose, 1.5 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 100 μg of $VB_1 \cdot HCl$, 3 μg of biotin, 350 mg of soybean hydrolysates, 3.0 g of urea and 0.02 ml of GD-113 in 1 L of pure water (adjusted to pH 7.0 with KOH) and cultured at 31.5°C with shaking. By referring to the method described in Journal of Fermentation and Bioengineering, Vol. 70, No. 3, 182-184, 1990, the GS activity was measured by adding a crude enzyme solution to a solution containing 100 mM imidazole-HCl (pH 7.0), 90 mM KCl, 0.1 mM NH_4Cl, 1 mM $MnCl_2$, 1 mM phosphoenolpyruvic acid, 0.3 mM NADH, 10 U of lactate dehydrogenase, 25 U of pyruvate kinase, 1 mM ATP and 10 mM MSG, and measuring variation of absorbance at 340 nm at 30°C. For the measurement of blank, the aforementioned reaction solution not containing MSG was used. The protein

concentration of the crude enzyme solution was quantified by using Protein Assay (Bio-Rad) with bovine serum albumin as a standard. It was verified that the GS activity was enhanced about three times in the GS activity-enhanced strain.

Table 6: GLS and GS activities of GLS-deficient strain and GLS-deficient and GS-enhanced strain

Strain	GLS activity (U/mg)	GS activity (U/mg)
ATCC 14067	0.19	0.019
2247Δgls	0.012	0.019
2247Δgls glnA14	0.014	0.063

(4) Evaluation of culture of GLS activity-reduced and GS activity-enhanced strain

By using the *Brevibacterium flavum* ATCC 14067 strain, 2247Δgls strain, and 2247Δgls glnA14 strain, culture for L-glutamine production was performed as follows. Cells of the *Brevibacterium flavum* ATCC 14067 strain, 2247Δgls strain, and 2247Δgls glnA14 strain obtained by culture on a CM2B plate medium were each inoculated into a medium containing 100 g of glucose, 60 g of $(NH_4)_2SO_4$, 2.5 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 350 μg of $VB_1 \cdot HCl$, 4 μg of biotin, 200 mg of soybean hydrolysates and 50 g of $CaCO_3$ in 1 L of pure water (adjusted to pH 6.8 with NaOH) and cultured at 31.5°C with shaking until the sugar in the medium was consumed.

After the completion of the culture, the amount of accumulated L-glutamine in the culture broth was analyzed for appropriately diluted culture broth by liquid chromatography. Furthermore, the accumulated L-glutamic acid amount was analyzed for appropriately diluted culture broth by using Biotech Analyzer AS210 (Asahi Chemical Industry). The results of the above analysis are shown in Table 7.

As for the 2247Δgls glnA14 strain, further improvement in the yield was recognized

compared with the 2247Δgls strain. These results demonstrated that, in addition to disappearance or reduction of the GLS activity, enhancement of the GS activity was effective for the production of L-glutamine.

Table 7: L-Glutamine production by GLS-deficient strain and GLS-deficient and GS-enhanced strain

Strain	OD_{620} (X101)	Gln (g/L)	Glu (g/L)	Gln yield (%)
ATCC 14067	0.469	13.4	35.2	13.8
2247Δgls	0.456	18.8	31.0	19.2
2247Δgls glnA14	0.436	24.4	26.0	24.9

Reference Example

(1) Measurement of glutaminase activity of known L-glutamine producing bacteria and and 2247Δgls glnA14

The strains AJ11576 and AJ11577 which were obtained as strains tolerant to a substance having vitamin P activity (Japanese Patent Laid-open No. 56-164792), AJ11573 and AJ11574 which were obtained as strains tolerant to α-ketomalonic acid (Japanese Patent Laid-open No. 56-151495), AJ12418 and AJ12419 which were obtained as strains tolerant to a peptide containing glutamic acid (Japanese Patent Laid-open No. 2-186994) and so forth are known as L-glutamine producing bacteria. The GLS activity of these L-glutamine producing bacteria and 2247Δgls glnA14 strains obtained in Example 4 was measured by the method described in Example 1. The results are shown in Table 8. All of these known L-glutamine producing strains had significant GLS activity.

Table 8: GLS activity of known L-glutamine producing bacteria and 2247Δgls glnA14

	Strain	GLS activity (U/mg)
Brevibacterium flavum	ATCC 14067	0.19

Corynebacterium glutamicum	ATCC 13032	0.17
Brevibacterium flavum	2247Δgls glnA14	0.010
Brevibacterium flavum	AJ11576	0.21
Corynebacterium glutamicum	AJ11577	0.14
Brevibacterium flavum	AJ11573	0.13
Corynebacterium glutamicum	AJ11574	0.18
Brevibacterium flavum	AJ12418	0.16
Corynebacterium acetoacidophilum	AJ12419	0.20

While the invention has been described in detail with reference to preferred embodiments thereof, it will be apparent to one skilled in the art that various changes can be made, and equivalents employed, without departing from the scope of the invention. Each of the aforementioned documents, and including the foreign priority JP2002-342287, is incorporated by reference herein in its entirety.